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Impact of Cytokines on Replication in the Thymus of Primary Human Immunodeficiency Virus Type 1 Isolates from Infants

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Early infection of the thymus with the human immunodeficiency virus (HIV) may explain the more rapid disease progression among children infected in utero than in children infected intrapartum. Therefore, we analyzed infection of thymocytes in vitro by HIV type 1 primary isolates, obtained at or near birth, from 10 children with different disease outcomes. HIV isolates able to replicate in the thymus and impact thymopoiesis were present in all infants, regardless of the timing of viral transmission and the rate of disease progression. Isolates from newborns utilized CCR5, CXCR4, or both chemokine receptors to enter thymocytes. Viral expression was observed in discrete thymocyte subsets postinfection with HIV isolates using CXCR4 (X4) and isolates using CCR5 (R5), despite the wider distribution of CXCR4 in the thymus. In contrast to previous findings, the X4 primary isolates were not more cytopathic for thymocytes than were the R5 isolates. The cytokines interleukin-2 (IL-2), IL-4, and IL-7 increased HIV replication in the thymus by inducing differentiation and expansion of mature $CD27^+$ thymocytes expressing CXCR4 or CCR5. IL-2 and IL-4 together increased expression of CXCR4 and CCR5 in this population, whereas IL-4 and IL-7 increased CXCR4 but not CCR5 expression. IL-2 plus IL-4 increased the viral production of all pediatric isolates, but IL-4 and IL-7 had a significantly higher impact on the replication of X4 isolates compared to R5 isolates. Our studies suggest that coreceptor use by HIV primary isolates is important but is not the sole determinant of HIV pathogenesis in the thymus.

The thymus is the essential site of normal T-cell development during fetal and neonatal life (reviewed in references 29 and 48). Therefore, human immunodeficiency virus (HIV) infection of the thymus in utero and in early life not only may have repercussions in situ but also may affect the overall course of disease in children (50). Involvement of the thymus in pediatric AIDS is suggested by histological studies showing thymic involution and HIV infection of thymocytes in fetuses and children and by anomalies in peripheral T-cell subset distribution in HIV-infected children (reviewed in reference 24). In addition, thymus volume and thymic output in HIV-infected children correlate with parameters of rapid disease progression and with increased immune reconstitution after antiretroviral therapy (16, 80, 82).

Surface expression of CD4 and of specific chemokine receptors allows HIV-1 entry into cells, but several reports indicate that HIV-1 primary isolates predominantly use CXCR4 and/or CCR5 as coreceptors (reviewed in references 3 and 47). Initial studies suggest that HIV variants that use CCR5 as a coreceptor (R5) are preferentially transmitted from mother to child, although cases of vertical transmission of variants using CXCR4 (X4) and of an HIV isolate using STRL33 (CXCR6) have also been reported (58). CXCR4 is widely expressed in the thymus, at levels which inversely correlate with CD3 expression, i.e., mature $CD3^{+high}$ thymocytes express lower levels of CXCR4 than immature $CD3^{+low}$ and $CD3^-$ thymocytes (4, 37, 56, 88). Although CCR5 is expressed at very low levels in thymocytes, we (56) and others (88) have directly demonstrated that this chemokine receptor is used for infection of thymocytes by the R5 laboratory isolates JR-CSF and BAL. This distribution of chemokine receptors in the thymus may explain the rapid cytopathic effect of X4 HIV isolates for thymocytes at different stages of maturation in vitro and in SCID-hu mice (2, 34, 71, 73). However, loss of $CD4^+$ thymocytes is also observed after infection with R5 isolates, although at a later time postinfection (32, 66). The chemokine receptors CCR3, CCR4, CCR8, CCR9, and CXCR6 are also expressed in the thymus and may be used as coreceptors for HIV, as recently reported for CCR8 (20, 30, 44, 45, 52, 87).

Recent data in the literature have shown that, despite age-related involution, thymic tissue may remain functional throughout life, even in the face of HIV infection and therefore could be manipulated for regenerating a diverse T-cell compartment (reviewed in reference 29). Consequently, there is a renewed interest in the role of factors that control thymopoiesis and affect thymic output. The cytokines interleukin-2 (IL-2), IL-4, and IL-7 are clearly important for survival, proliferation, and differentiation of distinct thymocyte subsets, although there is still controversy in the literature about their precise mechanisms of action in the human thymus (72, 76; reviewed in reference 57). In particular, a potential role for IL-7 in T-cell homeostasis has been proposed, given the correlation between circulating IL-7 levels and $CD4^+$ T lym-

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phopenia in HIV-infected patients (also in patients receiving cancer chemotherapy) (11, 21, 51, 70, 77). Notwithstanding their role in thymopoiesis, IL-2, IL-4, and IL-7, either alone or in combination, enhance HIV replication (74). We have previously shown that, in the thymus replication of the X4 and R5 laboratory viruses, NL4-3 and JR-CSF are differentially controlled by IL-2, IL-4, and IL-7 (73). The effect of these cytokines on the kinetics of viral replication was explained in part by their effect on the regulation of coreceptor expression in thymocytes (56). We found that IL-4 and, to a lesser degree, IL-2 and IL-7 increase CXCR4 surface expression levels in mature $CD3^{+high}$ thymocytes, whereas IL-2 and IL-4 synergize to increase the number of cells expressing CCR5 in this mature subpopulation (56). In addition to their role in the upregulation of chemokine receptor (6, 33, 55, 78), IL-2, IL-4, and IL-7 have been shown to provide signals that enhance postentry stages of viral replication (10, 12, 28, 77).

We have further investigated here the effect of the cytokines IL-2, IL-4, and IL-7 on the distribution and phenotype of thymocyte subsets and their impact on the replication of HIV. In contrast to other studies with HIV type 1 (HIV-1)-based vectors and/or laboratory viruses, we examined the impact of cytokines on the replication of clinically relevant HIV primary isolates from neonates. These isolates were obtained at birth, or as close as possible to birth, from 10 children born to HIV-1-seropositive mothers and defined by previously established criteria as infected in utero or intrapartum (7, 14, 83; Y. J. Bryson, K. Luzuriaga, J. L. Sullivan, and D. W. Wara, Letter, N. Engl. J. Med. **327:**1246-1247, 1992). These studies of maternal-fetal transmission showed that in most cases in utero-infected infants developed symptoms of HIV infection significantly earlier than did intrapartum-infected infants, prompting us to investigate whether virus isolates obtained from these infants differed in their ability to productively infect the thymus and disrupt thymopoiesis. We found that all 10 HIV pediatric isolates were able to replicate in thymocytes and used CXCR4 and/or CCR5 to enter thymocytes. The pathogenic effect of neonatal HIV isolates on CD4 expression and distribution in the thymus did not correlate with the clinical evolution of the children, nor their timing of infection. However, our studies showed that cytokines involved in T-cell development differentially affect the replication of HIV pediatric isolates in thymocytes by altering chemokine receptor expression in specific subsets.

MATERIALS AND METHODS

Reagents and MAbs. For thymocyte cultures, recombinant human IL-2 and IL-4 were provided by Amgen, Inc. (Thousand Oaks, Calif.). Recombinant human IL-7 was a gift from Immunex Corp. (Seattle, Wash.). For preparation of an allogeneic pool of $CD4^+$ T cells, recombinant human IL-2 from Chiron (Emeryville, Calif.) was used. Mitomycin C, hydroxyurea (HXU), and 7-amino-actinomycin D (7-AAD) were obtained from Sigma (St. Louis, Mo.), and actinomycin D was from Boehringer Mannheim (Indianapolis, Ind.). The bicyclam 1,1′-[1,4-phenylenebis-(methylene)]-bis-(1,4,8,11-tetrazacyclotetradecane) octahydrochloride dihydrate] (AMD3100) was kindly provided by G. Henson (AnorMED, Langley, British Columbia, Canada) and synthesized as previously described (8). Non-azide-purified unconjugated monoclonal antibodies (MAbs) to CXCR4 (12G5) and CCR5 (2D7), as well as the phycoerythrin (PE)-labeled MAbs were obtained from Pharmingen (San Diego, Calif.). MAbs to CD3, CD4, CD8, CD25, CD27, CD71, CD44, CD45RA, CD69, and HLA-DR conjugated with fluorescein (FITC) or PE were obtained from Becton Dickinson Immunocytometry Systems (BDIS; San Jose, Calif.). The MAb KC57-FITC, which identifies intracellular HIV p24 gag antigen expression, and CD1-PE (CD1a) were obtained from Coulter/Immunotech (Hialeah, Fla.). MAbs to CD3, CD4, and CD8 conjugated with Tricolor (Cy5-PE-tandem, referred to here as TC) were obtained from Caltag (Burlingame, Calif.).

TABLE 1. Characteristics of HIV-1 primary isolates from infants[a]

Isolate	Time of transmission[b]	Time of virus isolation[c]	Disease progression[d]	Syncytium induction[e]	Reference
PI-1	In utero	Birth	Rapid	+	14
PI-2	In utero	7 days	Rapid	+	15, 83
PI-3	In utero	Birth	Rapid	−	
PI-4	In utero	1 day	Rapid	−	
PI-5	In utero	60 days	Rapid	ND	14, 15, 23
PI-6	In utero	30 days	Rapid	−	14, 15, 23
PI-7	In utero	1 day	Slow	−	14
PI-8	Intrapartum	10 days	Slow	−	14, 15
PI-9	Intrapartum	90 days	Slow	−	23
PI-10	Intrapartum	18 mo	Slow	−	15, 23

[a] HIV isolates were obtained by coculture of blood mononuclear cells (children 1 to 4, 7, 8, and 10) with PHA-stimulated PBMC from seronegative donors. For children 5, 6, and 9 the isolation of HIV from plasma was done in purified $CD4^+$ cells.

[b] The timing of transmission was defined as "in utero" when HIV cocultures from PBMC were positive within 48 h of birth and/or positive by HIV DNA PCR. "Intrapartum" transmission was defined by HIV-negative cultures and negative DNA PCR within 48 h, followed by positive cultures within the first 90 days of life in non-breast-fed infants (Bryson et al., letter).

[c] In cases of intrauterine transmission, the virus isolate obtained within 48 h of birth was not always available for this study, and the virus isolate from the sample closest to birth was used. The time of isolation of the primary isolates used in this study is given as time after birth or "Birth" for at birth.

[d] Rapid disease progressors were defined as children with onset of at least two HIV-1-related symptoms (CDC class A, B, or C) within 6 months of birth, a significant fall in CD4 cell count within 1 year of birth, and onset of AIDS within 2 years of age. Slow progressors were defined as children with no HIV-1-related symptoms until >6 months of age, stable CD4 cell counts, and a lack of progression to AIDS (CDC class C) within the first 2 years of life.

[e] The ability to induce syncytia in MT-2 cells, indicated by a "+" sign, was assessed as previously described (38). PI-5 was not tested in this system; however, earlier isolates obtained from PBMC of the same infant but unavailable for this study did not induce syncytia in MT-2 cells. ND, not determined.

HIV pediatric isolates and virus stocks. HIV primary isolates and plasma samples for virus isolation were obtained as close as possible to birth from 10 of 34 children born to 204 HIV-1-seropositive mothers monitored prospectively in the Los Angeles Pediatric AIDS Consortium between June 1988 and May 1995 (23, 83; Y. J. Bryson et al., unpublished data). Table 1 summarizes the characteristics of the isolates available for this study. According to the proposed definition (Bryson et al., letter), the timing of transmission was defined as in utero when HIV cocultures from peripheral blood mononuclear cells (PBMC) were positive within 48 h of birth and/or were positive by HIV DNA-PCR. Negative cultures and HIV DNA-PCR within 48 h of birth followed by positive cultures within the first 90 days of life in non-breast-fed infants defined intrapartum transmission. Rapid disease progressors were defined as children with onset of at least two HIV-1-related symptoms (Centers for Disease Control and Prevention [CDC] classes A, B, or C) within 6 months of birth, a significant fall in CD4 cell count within 1 year of birth, and onset of AIDS within 2 years of age. Slow progressors were defined as children with no HIV-1-related symptoms until >6 months of age, stable CD4 cell counts, and a lack of progression to AIDS (CDC class C) within the first 2 years of life. The isolates were characterized as syncytium inducing (SI) or non-syncytium inducing (NSI) by using the MT-2 assay (38). The characteristics of some of the children providing the viral isolates were previously described: PI-2 is a SI HIV-1 isolate obtained 1 week after birth from an infant infected in utero with a SI isolate who developed AIDS and died within 6 months (83). The isolates PI-1, PI-5, PI-8, PI-6, and PI-7 were obtained from infants previously identified as patients 1, 8, 15, 17, and 28 (14). The viral isolates PI-5, PI-6, PI-9, and PI-10 were obtained from the original blood samples used to study the genetic diversity of HIV in children identified as A, B, C, and D, respectively (23).

HIV-1 isolation from plasma was obtained by directly adding plasma from infected children to a pool of purified activated allogeneic $CD4^+$ cells, prepared as described previously (19). Briefly, allogeneic $CD4^+$ cells from three normal donors were individually purified by capture in CD4 MAb-coated tissue culture flasks (Applied Immunosciences, Santa Clara, Calif.) and activated by stimula-

tion with antibody to CD3 (OKT3; Ortho Biotech, Inc., Raritan, N.J.) at 200 ng/ml and with recombinant IL-2 (5,000 U/ml) for 5 days. Cells from three donors were combined, cryopreserved in liquid nitrogen, and then thawed and cultured in medium with IL-2 for 2 to 3 days before infection. The same CD4 pool was used for isolating virus from plasma (passage 1) and for preparing virus stocks (passage 2). HIV isolates from blood samples were obtained by serial dilution of the child's mononuclear cells and coculture with phytohemagglutinin (PHA)-stimulated normal donor PBMC as previously described (14). Viruses recovered from the first coculture-positive well were expanded by a single passage in PHA-stimulated normal donor PBMC (passage 1). Virus stocks were prepared by passing the primary pediatric isolates (passage 1) once in the activated cryopreserved $CD4^+$ pool described above (passage 2). The makeup by phenotype of the $CD4^+$ pool at the time of infection by the pediatric isolates was as follows: 95% $CD4^+$ $CD8^-$, 5% $CD4^+$ $CD8^+$, <1% $CD8^+$ $CD4^-$, 60% $CD27^+$, and 50% HLA-DR^+. In addition, 94% of the cells from the $CD4^+$ stimulated pool expressed CXCR4 and 30 to 40% expressed CCR5 (data not shown).

The R5 virus HIV-$1_{JR\text{-}CSF}$ (JR-CSF) (40) and the X4 virus HIV-$1_{NL4\text{-}3}$ (NL4-3) (1), used as controls for these studies, were prepared as previously described, i.e., in PHA-stimulated PBMC and in CEM cells, respectively (56). No differences were found between these stocks and JR-CSF and NL4-3 stocks expanded in the cryopreserved $CD4^+$ T-cell pool (data not shown and Table 3). The infectivity of the pediatric isolates and of the molecular-clone-derived laboratory viruses was determined by limiting-dilution assays in PBMC. All thymocyte infections with pediatric isolates were standardized by using equal numbers of virus infectious units/10^7 cells.

Thymocyte cultures. Normal pediatric thymuses from HIV-seronegative children were obtained in the course of corrective cardiac surgery. Single-cell suspensions and nylon wool purification were done as previously described: thymocytes were cultured at 1×10^7 to 2×10^7 cells/ml in serum-free Iscove modified Dulbecco medium (Irvine Scientific, Santa Ana, Calif.) supplemented with delipidated bovine serum albumin (Sigma) at 1,100 μg/ml, transferrin (Sigma) at 85 μg/ml, 2 mM glutamine, and penicillin-streptomycin at 25 U/25 μg/ml (serum-free medium) (74). Nylon wool-purified thymocytes were incubated at 1×10^7 to 2×10^7 cells/ml as pellet cultures at 37°C in 5% CO_2 in round-bottom tissue culture tubes in the presence or absence of the following cytokines: IL-2 (20 U/ml), IL-4 (20 ng/ml), or IL-7 (200 U/ml [77 ng/ml]) singly or in combinations of IL-2 and IL-4 (IL-2+IL-4) or of IL-4 and IL-7 (IL-4+IL-7).

To block thymocyte proliferation in the presence of these cytokines, HXU or mitomycin C were used. In the first case, thymocytes were incubated with cytokines in the presence or absence of HXU (10 mM) throughout the experiment. In the second case, thymocytes were preincubated with or without mitomycin C at 25 μg/ml for 30 min at 37°C, washed several times, and cultured in the presence of the indicated cytokines.

For thymic organ cultures, a modification of the protocol of Galy et al. (22) was used; i.e., thymic pieces were excised from postnatal specimens and cultured on floating rafts consisting of size four absorbable gelatin sponges (Gelfoam; Pharmacia and Upjohn, Kalamazoo, Mich.) covered with 0.8-μm (pore-size) nitrocellulose disks (MSI, Westboro, Mass.). Thymic organ cultures were maintained at 37°C in 5% CO_2 in six-well plates containing serum-free medium supplemented with cytokines.

Thymocyte infection and blocking studies with antibodies to chemokine receptors and AMD3100. Thymocytes were infected and cultured as previously described (56, 74). Briefly, 10^7 freshly isolated, nonstimulated thymocytes were incubated with 30 to 50 ng of viral p24 (multiplicity of infection [MOI] = 0.01) in the presence of 10 μg of Polybrene (Sigma)/ml for 1 to 2 h at 37°C. NL4-3 was used at a 10- to 20-fold-lower MOI than the pediatric isolates and JR-CSF unless otherwise indicated. Control thymocytes were mock infected in the presence of Polybrene with supernatants from the same uninfected cells used to prepare the virus stocks. After infection, the cells were washed and resuspended in serum-free medium in the presence of cytokines. To investigate coreceptor use, thymocytes were preincubated with the CXCR4-specific antagonist AMD3100 (1 μg/10^7 cells) or antibodies to CCR5 (2D7; 5 μg/10^7 cells) and/or to CXCR4 (12G5; 10 μg/10^7 cells) at 4°C for 2 h before infection. The antibodies and/or AMD3100 were present during infection and throughout the duration of the experiment. On day 1, and weekly thereafter, the medium was removed and fresh medium containing cytokines and the antibody or AMD3100 was added. As internal controls for the specificity of the inhibitory effects, thymocytes were infected in parallel with the molecularly cloned viruses NL4-3 and JR-CSF in the presence or absence of the antibodies to the coreceptors and/or AMD3100. Virus replication was assessed by measuring p24 antigen in the supernatant by a specific p24 antigen enzyme-linked immunosorbent assay (Coulter).

For infection of thymic organ cultures, thymus pieces were excised, pooled in a 15-ml conical tube, and incubated for 2 h with virus- or mock-infected supernatants in the presence of Polybrene. After several gentle washes in medium without cytokines, four pieces of infected or mock-infected organ tissue were returned to each duplicate Gelfoam raft, which had been preincubated for 1 h in medium with different cytokines at 37°C and in 5% CO_2. The medium with cytokines was changed at day 1 postinfection and every 2 days thereafter. Viral replication was assessed in the supernatant of individual wells by measuring the level of p24 antigen.

Immunofluorescent staining and flow cytometry. Dual-surface immunophenotyping of thymocytes with directly FITC- and PE-conjugated MAbs was done as previously described (62, 63). To exclude dead cells from the analysis, the thymocytes were incubated in a solution of 2 μg of 7-AAD/ml in phosphate-buffered saline (PBS) for 20 min at 4°C, washed in PBS, and resuspended in 1% paraformaldehyde solution in PBS containing 4 μg of actinomycin D/ml (63). To determine HIV expression in distinct thymocyte subsets, surface staining of thymocytes was performed with PE- and TC-conjugated antibodies, followed by fixation in paraformaldehyde, permeabilization in 0.2% Tween, and cytoplasmic staining for HIV gag antigen with the FITC-conjugated KC57 MAb, as previously described (62, 73). To assess intracellular expression of CXCR4, thymocytes were stained with CD3-FITC, fixed and permeabilized as described above, and then stained with CXCR4-PE. To compare internal and surface expression of CXCR4, we assessed CXCR4 surface expression in parallel by using the methods for intracellular staining; i.e., thymocytes were stained with CD3-FITC and CXCR4-PE and fixed and permeabilized as described above. A FACScan flow cytometer equipped with a standard filter setup (BDIS) was used in these experiments. A minimum of 10,000 events in the live cell population was acquired of each sample stained with 7-AAD. Multiparameter data acquisition and analysis were performed with CellQuest software (BDIS).

Statistical methods. Mixed-effect linear models, fit by using the MIXED procedure in SAS version 8.2, were used for all statistical inference (46). These models were used to account for the dependence in measured quantities due to (i) repeated measurements on the same viral isolate over time within an experiment and (ii) correlation between isolates tested in thymocytes from a single donor. Specifically, the kinetics of viral replication among different pediatric viral isolates were studied by using a model that allowed isolate-specific linear trajectories of the logarithm of p24 level. Comparisons were made by considering the estimated mean value of p24 at approximately 2 weeks postinfection. Assessment of the inhibition of viral replication by chemokine receptor antagonists (antibodies to CCR5 or AMD3100) was performed in a similar manner. To determine the differences in the levels of CD4 expression among thymocytes infected with pediatric HIV isolates and molecularly cloned isolates, taking into account the intraexperimental correlations as explained above, we evaluated the percentages of CD4 and CD8 subsets at 2 weeks postinfection in mock-infected and HIV-infected thymocytes.

Human subjects. All human studies were approved by the UCLA Office for Protection of Research Subjects.

RESULTS

HIV-1 pediatric isolates from infants productively infect thymocytes. The HIV isolates described in Table 1 were used to infect freshly isolated, nonstimulated, thymocytes which were then cultured for 2 to 4 weeks in serum–free medium with IL-2+IL-4. This combination of cytokines favors the replication of the molecular clones JR-CSF and NL4-3 in vitro. We found that all 10 pediatric HIV-1 isolates were able to productively infect thymocytes, with viral antigen (p24) reproducibly detected in the supernatant of the cultures at 2 to 3 weeks postinfection (Fig. 1 and data not shown). Since variation in the amount of p24 produced is observed when thymocytes from different donors are infected with the molecularly cloned derived laboratory viruses NL4-3 and JR-CSF, each HIV pediatric isolate was used to infect thymocytes from at least three different donors. In addition, each pediatric isolate was tested in parallel with one or both laboratory viruses at least once. Pairwise comparisons were established to assess if the replication kinetics of these HIV isolates in the thymus correlated with the rate of disease progression in the children or their

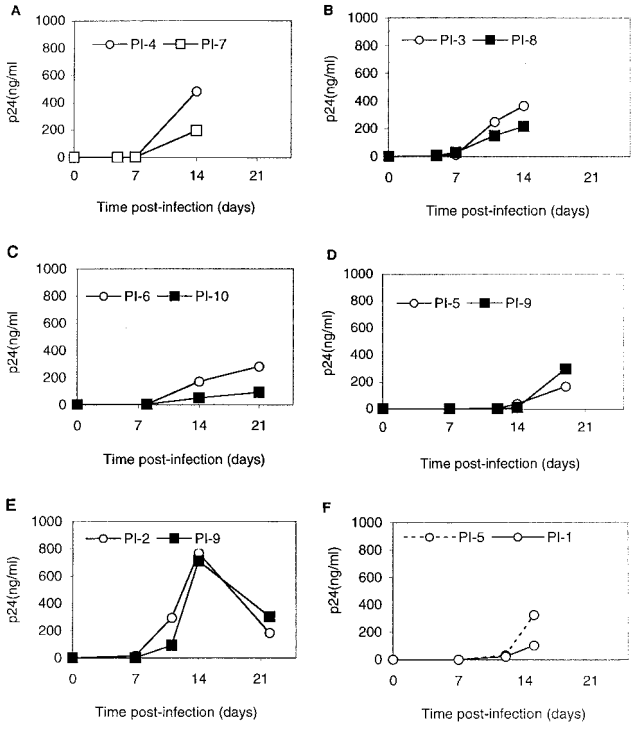

FIG. 1. HIV-1 primary isolates from neonates and infants productively infect thymocytes. Freshly isolated thymocytes were infected in vitro with HIV pediatric primary isolates and cultured for 2 to 3 weeks with IL-2 (20 U/ml) and IL-4 (20 ng/ml) (IL-2+IL-4). Viral production was measured by p24 antigen levels in the supernatant. Each HIV pediatric isolate described in Table 1 was used to infect at least three different thymus specimens from HIV-seronegative children. Each panel represents an independent experiment in which thymocytes from a given donor were infected with a pair of pediatric isolates. Thymocytes were infected in parallel with a pair of HIV isolates from infants with rapid (circles) or slow (squares) rate of disease progression. (A) Isolates from children infected in utero (open symbols) with different rate of disease progression. (B, C, D, and E) Isolates from children with rapid disease progression infected in utero (○) compared to early isolates from children with slow disease progression infected intrapartum (■). (F) Replication kinetics of two HIV isolates from neonates infected in utero with rapid disease progression.

timing of infection. The results were analyzed by using the statistical methods described above. Figure 1 shows six different representative experiments (in panels A to F) wherein thymocytes from six different donors were infected in parallel with pairs of HIV isolates from children with different rates of disease progression (slow versus rapid, Fig. 1A to E) and/or timing of transmission (intrapartum versus in utero, Fig. 1B to E). Panels A and F show two experiments in which the kinetics of replication of HIV isolates from children with similar mode of transmission were compared. The difference between the rate of replication of HIV pediatric isolates from children with rapid versus slow disease progression in thymocytes was not statistically significant, i.e., the estimated p24 production at the mid-point of the growth curves (approximately 2 weeks postinfection) was nearly identical for both groups (average 17.2 and 18.4 ng/ml, respectively; $P = 0.7$). Neither was the difference between the kinetics of replication of HIV isolates from children infected in utero versus intrapartum (20.1 and 16.7, respectively; $P = 0.3$; see also Fig. 5).

AMD3100 inhibits the replication of a CXCR4-tropic molecular clone in thymocytes without interfering with T-cell development in vitro. In order to determine the use of coreceptors by the HIV pediatric isolates directly in thymocytes rather than in coreceptor-transfected cell lines, we needed blocking agents for CCR5 and CXCR4 that also efficiently and specifically blocked viral replication. Since AMD3100 induces conformational changes in CXCR4 and specifically blocks HIV entry into the cells via this coreceptor (25, 64, 65), we evaluated the activity of this compound in the thymus and its effect on T-cell development. We found that AMD3100 at 1 μg/ml was more effective in blocking replication of the X4 virus NL4-3 than the MAb 12G5 (data not shown). CXCR4 surface expression was decreased in the $CD3^{+high}$ population in which we found upregulation of CXCR4 by the cytokines IL-2, IL-4, and IL-7 (56). This is probably due to the fact that AMD3100 disrupts the conformational site recognized by the antibody 12G5; as expected, intracellular expression of CXCR4 was not affected by AMD3100 (Table 2). We found no differences in the distribution of thymocyte subsets when AMD3100 was added in the presence of cytokines (Table 2) or in thymocytes cultured with AMD3100 in the absence of cytokines (data not shown).

Use of the coreceptors CXCR4 and CCR5 in thymocytes by HIV primary isolates from infants. Blocking studies with the CCR5 MAb 2D7 and AMD3100 were performed in the presence of IL-2+IL-4 to assess whether the HIV pediatric isolates used CCR5, CXCR4, or alternate coreceptors in the thymus (Fig. 2). An overview of the effect of these chemokine receptor

TABLE 2. Blocking of CXCR4 surface expression on thymocytes by AMD3100[a]

Antagonist	% Thymocyte subset[b]					% Surface CXCR4 (MFI)[c]		% Intracellular CXCR4 (MFI)[c]	
	$CD4^+$ $CD8^+$	CCR5	CD1	CD27	CD45RA	Total cells	$CD3^{+high}$ cells	Total cells	$CD3^{high}$ cells
None	55	3	79	19	12	98 (232)	25 (509)	59 (115)	22 (136)
AMD3100	58	4	74	21	13	82 (80)	9 (31)	62 (106)	21 (123)

[a] Thymocytes were preincubated in serum-free medium for 2 h at 4°C in the presence or absence of AMD3100 at 1 μg/ml/10^7 cells and then cultured for 2 weeks in the presence of IL-2+IL-4 with or without AMD3100, respectively.

[b] That is, the effect of AMD3100 in the thymus. The surface expression of thymocyte development markers was assessed with the following antibodies: CD1-PE, CCR5-PE, CD4-PE, CD8-FITC, CD27-FITC, and CD45RA-FITC. 7-AAD was used to exclude dead cells. Gates were set on of live and early apoptotic cells. The percentages of thymocytes expressing the respective surface antigen in the total cell population are shown.

[c] That is, the expression of CXCR4 in the total population and in the $CD3^{+high}$ population. The surface expression of CXCR4 and CD3 was assessed by using methods for intracellular staining, i.e., live cells were stained with CXCR4-PE and CD3-FITC, fixed, and permeabilized prior to acquisition. The intracellular expression of CXCR4 was assessed in parallel, after fixation and permeabilization of cells previously stained with CD3-FITC only. The appropriate isotype controls were used for setting cursors; $CD3^{+high}$ cells were defined by assessing the surface CD3 expression in cells stained with CD3-FITC only. The mean fluorescence intensity (MFI) of CXCR4 in the total cell population or in the $CD3^{+high}$ cells was calculated with CellQuest software and is indicated in parentheses.

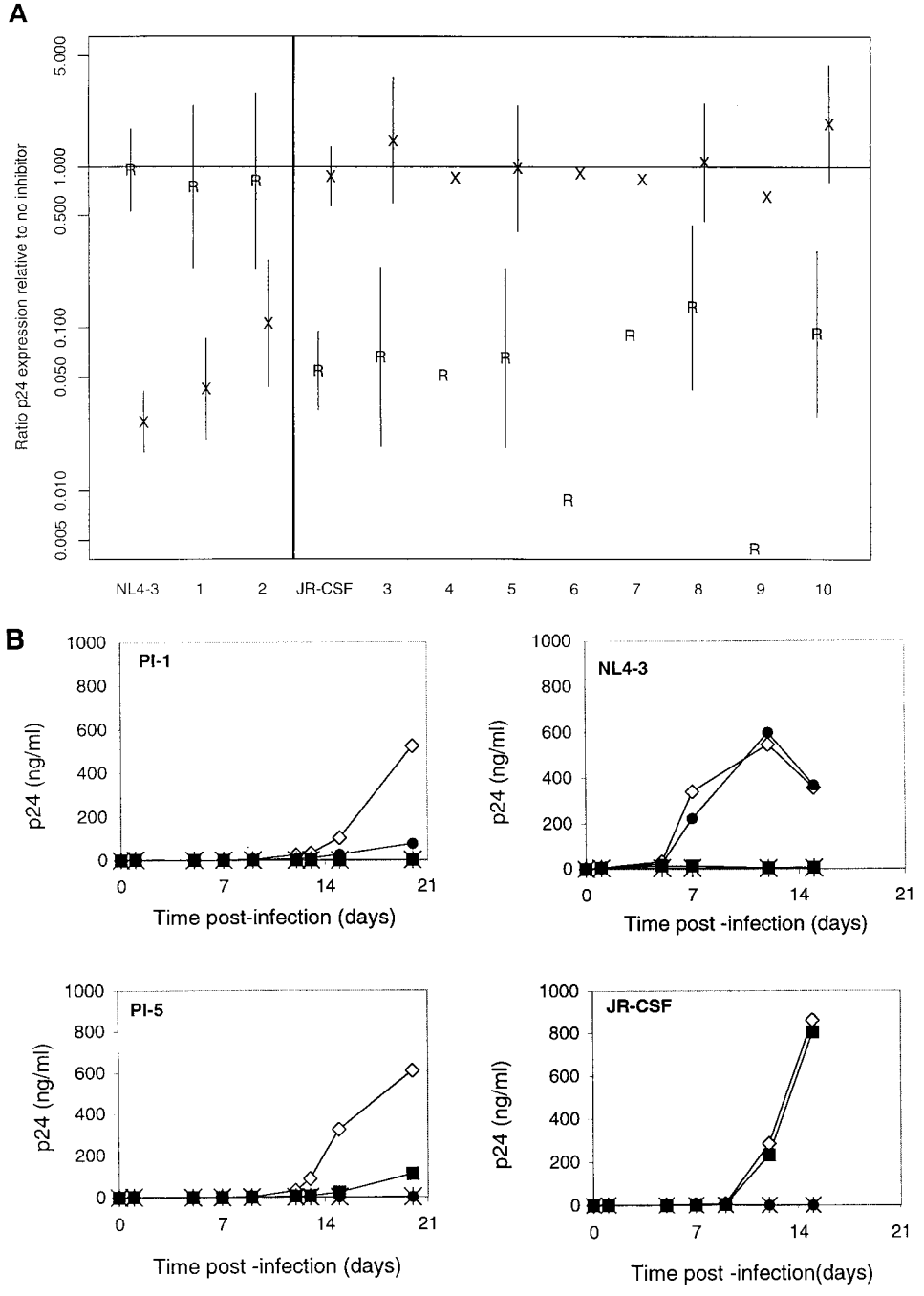

FIG. 2. HIV isolates from neonates and infants use CXCR4, CCR5, or both chemokine receptors as coreceptors in thymocytes. Thymocytes were preincubated at 4°C in serum-free medium without cytokines with or without the specific coreceptor inhibitors AMD3100 (CXCR4) at 1 μg/ml and the 2D7 antibody to CCR5 (CCR5 MAb) at 5 μg/ml. In each experiment, thymocytes were infected with the pediatric isolates (MOI = 0.01), the X4 virus NL4-3 (MOI = 0.001), or the R5 virus JR-CSF (MOI = 0.01) and then cultured with IL-2+IL-4 and the indicated blocking agents for 2 to 3 weeks. (A) Inhibition of virus production at the peak of infection. The estimated inhibition ratios of the blocking agents are indicated for each pediatric isolate, NL4-3, and JR-CSF. X, AMD3100; R, MAb 2D7 for CCR5. The intervals provide 95% confidence limits for these estimated ratios that take into account all experiments involving each isolate. (B) Kinetics of replication of HIV isolates in the presence of chemokine receptor inhibitors. Thymocytes were infected in parallel with isolates PI-1, PI-5, NL4-3, and JR-CSF. ◇, No inhibitors; ●, CCR5 MAb; ■, AMD3100; ×, AMD3100 plus CCR5 MAb.

blocking agents on the replication of the 10 pediatric isolates in thymocytes is shown in Fig. 2A. At 2 weeks postinfection, the replication of all but one (PI-8) HIV pediatric isolate was completely inhibited when one of the blocking agents was present, suggesting preferential use of CCR5, CXCR4, or both chemokine receptors by the HIV isolates from infants to enter thymocytes in vitro. Among the infants with rapid disease progression, three of six had HIV variants able to use CXCR4 in

the thymus, whereas none of the four isolates obtained from children with slow disease progression used CXCR4 as coreceptor. However, R5 isolates, which replicated at high levels in the thymus were present at or close to birth in three of six infants who rapidly progressed to AIDS. This suggests that rapid disease progression in children infected perinatally could not be ascribed exclusively to the presence of X4 isolates at birth (Fig. 2A and Table 1). Indeed, among the seven isolates from children infected in utero, only the SI isolate PI-2 was exclusively blocked by AMD3100 in thymocytes (Fig. 2A). Replication in the thymocytes of two isolates (PI-1 and PI-5) was totally inhibited by one of the blocking agents and partially inhibited by the other agent (AMD3100 and antibody to CCR5, respectively), as shown in Fig. 2B. The partial blocking of virus replication was not due to nonspecific inhibitory effects of the antibody to CCR5 or of AMD3100, since they did not block replication of the X4 virus NL4-3, or of the strictly R5 virus JR-CSF, respectively. Furthermore, replication of the pediatric isolates was completely inhibited when both coreceptor inhibitors were present, suggesting that they used those coreceptors rather than alternative coreceptors to infect thymocytes (Fig. 2B). However, PI-1 and PI-5 were not able to use CXCR4 and CCR5 with the same efficiency, since inhibition of virus replication by one of the chemokine receptor antagonists was not overcome by the ability of the isolate to use the other coreceptor. It is not clear whether these HIV primary isolates, which were not biologically cloned, were composed of a major variant able to use both coreceptors (X4R5) or of multiple variants able to use one or the other coreceptor (X4+R5).

Tropism of HIV-1 isolates from infants in the thymus. We next examined whether the pediatric isolates from children with rapid or slow rates of disease progression differed in their abilities to replicate in distinct thymocyte subsets and impact thymopoiesis. Thymocytes were characterized at the time of infection and at various times after infection with the distinct isolates for the presence of developmental markers such as CD3, CD1, CD27, and CD45RA in combination with intracellular staining for HIV gag proteins (Fig. 3 and data not shown). These cell surface molecules were previously shown to distinguish thymocyte subsets able to support productive infection of HIV laboratory isolates (56, 75; K. B. Gurney and C. H. Uittenbogaart, unpublished observations). We found that the tropism of the HIV pediatric isolates for thymocytes subsets was directly correlated with coreceptor use: the PI-1 and PI 2 isolates, which use CXCR4 as their main coreceptor, were detected in $CD1^+$ and $CD1^-$ cells (Fig. 3A), whereas R5 pediatric isolates and the R5X4 isolate PI-5 were present only in mature $CD1^-$ cells. Further characterization of this subset showed that expression of the R5 pediatric isolates was restricted to the mature $CD3^{+high}$ $CD27^+$ thymocytes. A representative experiment is shown in Fig. 3B.

Pediatric isolates are cytopathic for discrete thymocyte subsets. To assess the impact of the pediatric isolates on T-cell development, we determined the surface phenotype of virus-infected cells vis-a-vis mock-infected thymocytes cultured in the presence of the cytokines IL-2 plus IL-4. All pediatric isolates were analyzed in at least three independent experiments. On average, pediatric isolates induced a statistically significant decrease in the total $CD4^+$ population ($P = 0.0001$), the $CD4^+$ $CD8^-$ ($P = 0.0001$) and $CD4^+$ $CD8^+$ ($P = 0.0001$) populations compared to their respective mock-infected controls (Table 3). Similar decreases were observed when we compared CXCR4-using pediatric isolates or R5 pediatric isolates to their respective mock-infected controls in the three thymocyte populations. There were no statistically significant differences ($P = 0.5$) in the mean percentages of the CD4-bearing thymocytes when pediatric isolates obtained from infants with rapid disease progression were compared to those of patients with a slow disease progression. Nor were there differences in the mean percentages of the CD4-bearing cells ($P = 0.3$) between isolates obtained from infants infected in utero compared to those infected intrapartum (Table 3).

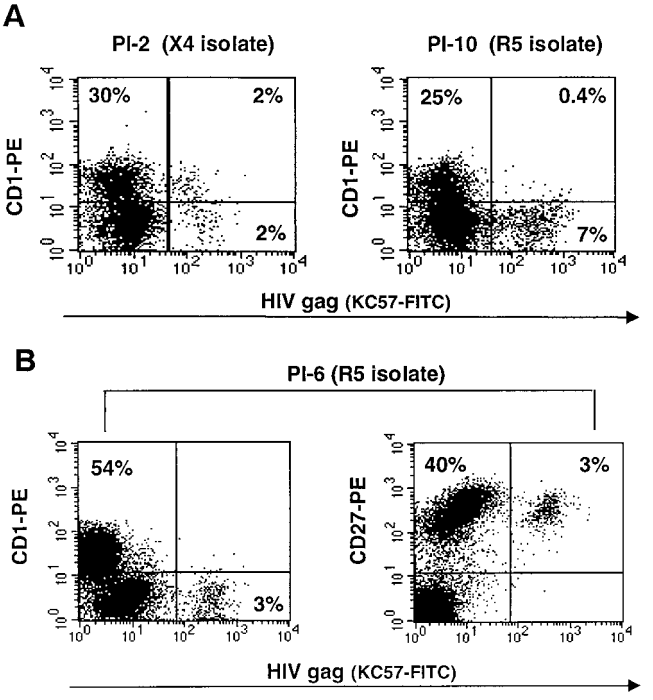

FIG. 3. X4 and R5 HIV pediatric isolates are expressed in thymocytes at different stages of maturation. Thymocytes were infected or mock infected with HIV pediatric isolates and then cultured for 2 weeks in the presence of IL-2+IL-4. Dual staining for surface thymocyte markers was done with MAb CD1-PE or CD27-PE and CD3-TC, followed by intracellular staining for HIV-1 gag antigen with the antibody KC57-FITC. Cursors were set by using isotype controls; HIV gag antigen was below detection in mock-infected cells. (A) Thymocytes were infected in parallel with isolates PI-2 or PI-10 and stained at 2 weeks postinfection. (B) The R5 isolate PI-6 was obtained directly from plasma, and first-passage virus was used to infect thymocytes. The effect of PI-6 on the distribution of thymocyte subsets in the same experiment is shown in Fig. 4B.

Despite the wider distribution of CXCR4 than CCR5 in the thymus, no differences were found between the CXCR4-using and R5 pediatric isolates in terms of the average percentage of total $CD4^+$ ($P = 0.8$), $CD4^+$ $CD8^-$ ($P = 0.8$), and $CD4^+$ $CD8^+$ ($P = 0.8$) populations (Table 3 and Fig. 4). We found that thymocytes infected with the CXCR4-using isolates PI-1 and PI-2, which induce syncytia in MT-2 cells and PBMC, had significantly higher percentages of total CD4 ($P = 0.0001$), $CD4^+$ $CD8^-$ ($P = 0.001$), and $CD4^+$ $CD8^+$ ($P = 0.0001$) cells than those infected with the laboratory virus NL4-3 (Fig. 4A). There was no significant difference between JR-CSF and R5

TABLE 3. CD4 expression on thymocyte subsets after infection with pediatric and laboratory HIV-1 isolates (including statistical analysis)[a]

Group		% $CD4^+$ cells (total)			% $CD4^+$ $CD8^-$ cells			% $CD4^+$ $CD8^+$ cells		
A	B	Mean A (SD)	Mean B (SD)	*P*	Mean A (SD)	Mean B (SD)	*P*	Mean A (SD)	Mean B (SD)	*P*
Mock (PI)	PI (All)	72.5 (9.5)	50.5 (15.1)	0.0001	20.9 (7.1)	11.0 (4.6)	0.0001	51.6 (9.8)	39.8 (13.1)	0.0001
Mock (PI)	X4 PI	70.2 (9.2)	46.0 (16.1)	0.001	22.4 (10.4)	10.6 (4.4)	0.001	47.9 (12.9)	35.5 (15.2)	0.01
Mock (PI)	R5 PI	73.3 (9.6)	52.2 (14.6)	0.0001	20.4 (5.7)	11.2 (4.8)	0.0001	52.9 (8.3)	41.4 (12.1)	0.0001
Rapid	Slow	49.2 (16.6)	52.3 (13.2)	0.5	11.2 (4.6)	10.8 (4.7)	0.4	38.0 (14.4)	42.0 (11.2)	0.7
IP	IU	50.3 (13.2)	50.7 (16.3)	0.3	10.3 (4.7)	11.4 (4.6)	0.3	40.7 (10.9)	39.2 (14.4)	0.5
X4 PI	R5 PI	46.0 (16.1)	52.2 (14.6)	0.8	10.6 (4.4)	11.2 (4.8)	0.8	35.5 (15.2)	41.4 (12.1)	0.8
X4 PI	NL4-3	46.0 (16.1)	14.9 (6.7)	0.0001	10.6 (4.4)	4.9 (4.2)	0.001	35.5 (15.2)	10.1 (3.9)	0.0001
R5 PI	JR-CSF	52.2 (14.6)	56.0 (8.0)	0.6	11.2 (4.8)	12.4 (4.1)	0.4	41.4 (12.1)	43.7 (7.3)	0.9
Mock (NL)	All NL4-3	72.1 (7.8)	14.9 (6.7)	0.0001	23.7 (8.0)	4.9 (4.2)	0.0001	47.9 (8.3)	10.1 (3.9)	0.0001
Mock (NL)	NL (CEM)	71.1 (8.3)	16.3 (7.5)	0.0001	20.5 (5.5)	5.6 (4.9)	0.001	50.5 (6.0)	10.7 (4.1)	0.0001

[a] Thymocytes were infected with pediatric isolates (PI), NL4-3 (NL), and JR-CSF and cultured in IL-2+IL-4. At 2 weeks postinfection, infected and mock-infected thymocytes were stained for CD4 and CD8. Apoptotic and dead cells were excluded by using 7-AAD, and quadrants were set by using the isotype controls. CellQuest software was used to analyze the data from HIV- and mock-infected thymocytes in all experiments. Statistical comparisons were made between mock- and HIV-infected thymocytes or according to the timing of transmission (intrapartum [i.p.] or intrauterine [i.u.]), rate of disease progression, and coreceptor use, defined as in Table 1. For each comparison of two groups, the mean and standard deviation for percentages of thymocyte subsets, and a *P* value for the difference in these means is shown. The *P* values were computed by using mixed-effect linear models in order to correctly account for intraexperimental correlations. NL4-3 prepared in CEM cells and NL4-3 prepared in the CD4 T-cell pool were compared to their respective mock-infected controls. For comparison of NL4-3 and X4 pediatric isolates, all experiments were computed. Mean A, mean for group A; Mean B, mean for group B.

pediatric isolates ($P = 0.6$, $P = 0.4$, and $P = 0.9$, respectively) (Table 3).

Figure 4B shows an example of the impact of two R5 isolates on thymocyte subsets. Compared to mock-infected thymocytes, the CD4 mean fluorescence intensity (MFI) of both mature $CD4^+$ $CD8^-$ and immature $CD4^+$ $CD8^+$ populations was significantly decreased by 35 and 40%, respectively ($P = 0.0001$ for both comparisons), after infection with R5 pediatric isolates. In addition, we found lower percentages of the mature $CD27^+$ $CCR5^+$ population, compared to mock-infected cells, 2 weeks after infection of thymocytes with these R5 isolates. In the experiment shown in Fig. 4B, thymocytes were infected with PI-9, an isolate from a child with slow rate of disease progression, and isolate PI-6, obtained from an infant with rapid disease progression. As mentioned above for the differences between NL4-3 and CXCR4-using pediatric isolates, there was a wide range of CD4 expression among thymocytes infected with the different R5 isolates that could not be ascribed to thymus donor variation. Small differences in the percentages of CD4-bearing cells were found between mock-infected and PI-4-infected thymocytes in three independent experiments, whereas PI-8-infected cells showed greater levels of depletion in seven independent experiments. Therefore, both R5 and X4 pediatric isolates were able to induce significant loss of CD4 expression in specific thymocyte subsets, and the impact of HIV infection in the thymus was not strictly dependent on coreceptor use.

Differential effect of IL-4 and IL-7 on HIV replication in the thymus. In contrast to the ability of all 10 pediatric isolates to productively infect thymocytes cultured in the IL-2+IL-4 combination after infection (Fig. 1 and Fig. 5), we found that pediatric HIV isolates differed in their response to the cytokines IL-4 and IL-7 (Fig. 5). HIV isolates from rapid disease progressors expressed more p24 than HIV isolates from slow disease progressors at 2 weeks postinfection ($P < 0.05$) (Fig. 5C). However, this was due to the fact that CXCR4-using clinical isolates had more rapid kinetics of replication than R5 isolates ($P < 0.0001$) in IL-4+IL-7 (Fig. 5D). Indeed, when the CXCR4-using isolates were excluded from the group of rapid disease progressors no differences were found between R5 isolates obtained from rapid and slow disease progressors in IL-4+IL-7.

The differences in replication of X4 and R5 isolates in IL-4+IL-7 could be due to the presence of other HIV coreceptors, which could be used by the X4 and R5X4 isolates but not by the R5 isolates. To address this issue, thymocytes were preincubated with specific inhibitors of CXCR4 and CCR5; infected with representative X4, R5X4, and R5 isolates; and cultured for 3 weeks in the presence of IL-4+IL-7 or IL-2+IL-4. As observed when blocking experiments were performed in the presence of IL-2+IL-4, replication of the X4 isolate PI-2 was blocked only by AMD3100, whereas the replication of the R5 isolate PI-8 was inhibited by antibody to CCR5 (2D7) and not by AMD3100 (data not shown).

To determine whether the combination IL-4+IL-7 was necessary for increased expression of X4 isolates in thymocytes, we assessed the effects of IL-4 and IL-7, individually as well as in combination, on the replication of a representative X4 isolate (Fig. 6). Both cytokines were able to induce an increase in p24 production when used independently (Fig. 6A). Parallel treatment of JR-CSF-infected thymocytes with IL-4 or IL-7 singly did not lead to enhanced virus production, as we have shown previously (74). Figure 6B shows that exogenous cytokines were also able to enhance the replication of the isolate PI-2 in thymic organ cultures, where stromal cells secreting IL-7 and other survival factors are present. This suggests an additional role for cytokines such as IL-4 and IL-7 on HIV replication beyond their ability to increase thymocyte viability in suspension cultures (data not shown).

Determinants of the differential effect of cytokines on HIV replication. We then examined whether the differential kinetics of replication of HIV primary isolates in response to cytokines in the thymus was solely linked to the effect of cytokines on coreceptor expression and/or their effect on the activation and proliferation state of specific thymocyte subsets. Thymocytes were assessed for CXCR4 and CCR5 expression and specific thymocyte activation and maturation markers before and after culture in the presence of cytokines and of the pro-

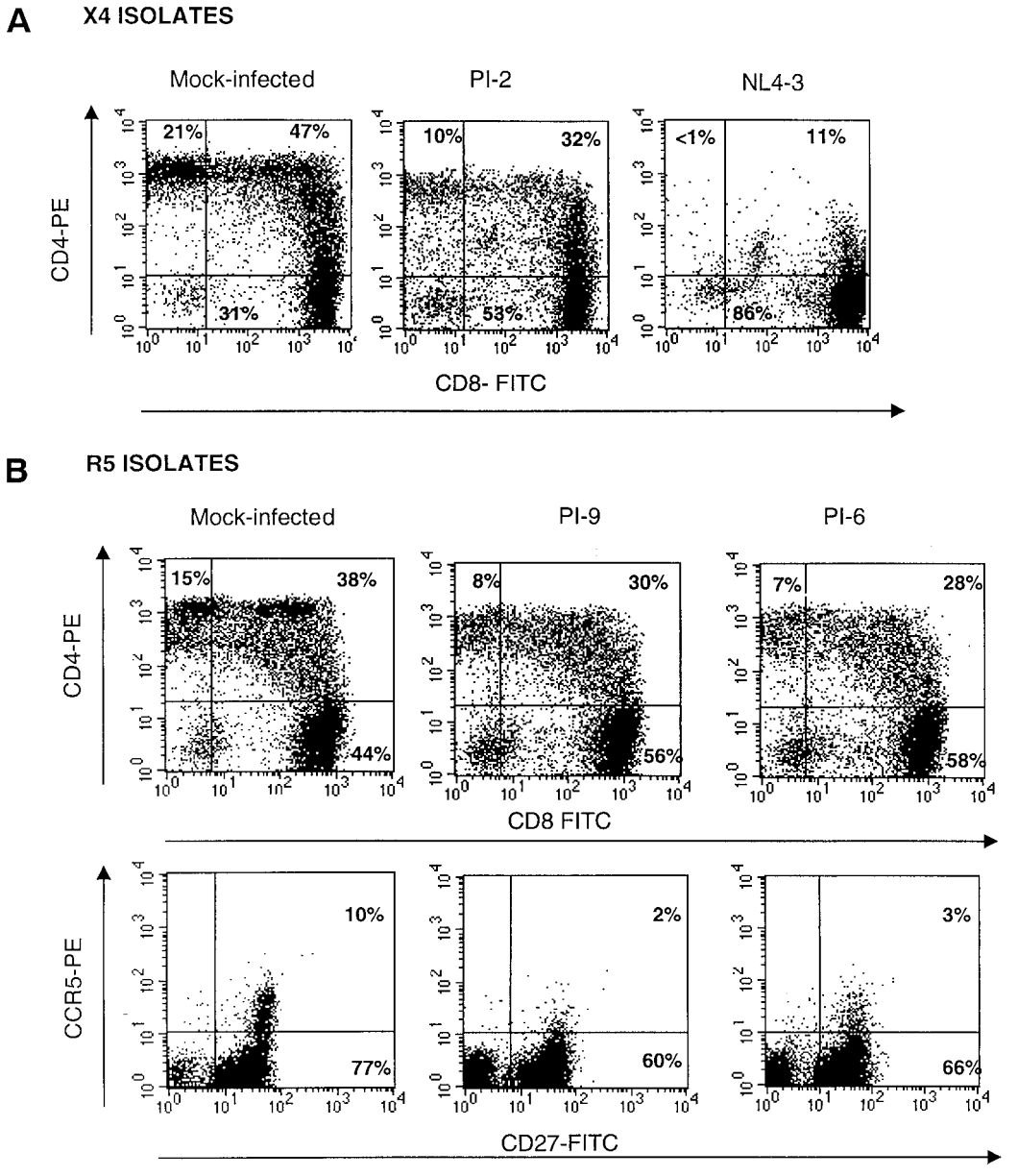

FIG. 4. Cytopathic effect of R5 and X4 primary isolates from infants in the thymus. Thymocytes were infected with pediatric isolates and cultured in the presence of IL-2+IL-4. At 2 and 3 weeks postinfection, infected and mock-infected thymocytes were stained for CD4, CD8, CCR5, and CD27 expression. Apoptotic and dead cells were excluded from the analysis by 7-AAD staining. (A) The X4 isolate PI-2 was used at an MOI of 0.01, and the NL4-3 laboratory virus was used at an MOI of 0.001. Thymocyte phenotype was examined at 3 weeks postinfection, when the p24 concentrations in the supernatant of thymocytes infected with PI-2 and NL4-3 were 275 and 145 ng/ml, respectively. (B) Thymocytes were infected with the R5 pediatric isolates PI-6 and PI-9 at the same MOI (0.01). Infected and mock-infected thymocytes were stained at 2 weeks postinfection, when the p24 concentrations were 200 ng/ml for both isolates.

liferation inhibitors HXU or mitomycin C (Table 4). Our results show that IL-4 and IL-7, either alone or in combination, provided signals that allow the in vitro maturation, expansion, and/or selective survival of mature $CD27^+$ and $CD45RA^+$ cells expressing high levels of CXCR4 compared to cells cultured in medium without cytokines. The number of cells expressing high levels of CXCR4 ($CXCR4^{+high}$) after incubation with IL-4 or IL-7 was decreased in the presence of cell cycle inhibitors (see the decrease in the percentage but not in the CXCR4 MFI in Table 4). These results indicate that, in addition to their effect on T-cell differentiation and CXCR4 upregulation, IL-4 and IL-7 induced the proliferation of subsets of mature $CD27^+$ $CXCR4^{+high}$ and $CD45RA^+$ $CXCR4^{+high}$ cells. In contrast, when proliferation was blocked in cells cultured in the presence of IL-2 alone, an increase in the CXCR4 MFI was observed in the mature populations (Table 4). This reflects the fact that IL-2 induces the proliferation of mature cells that do not express high levels of CXCR4 such as $CD27^+$ $CXCR4^{+low}$, $CD27^+$ $CXCR4^-$, and $CD45RA^+$ $CXCR4^-$ cells (data not shown). Figure 7A shows that upregulation of CXCR4 expression in the presence of IL-2+IL-4 occurred independently of proliferation, although the percentage of

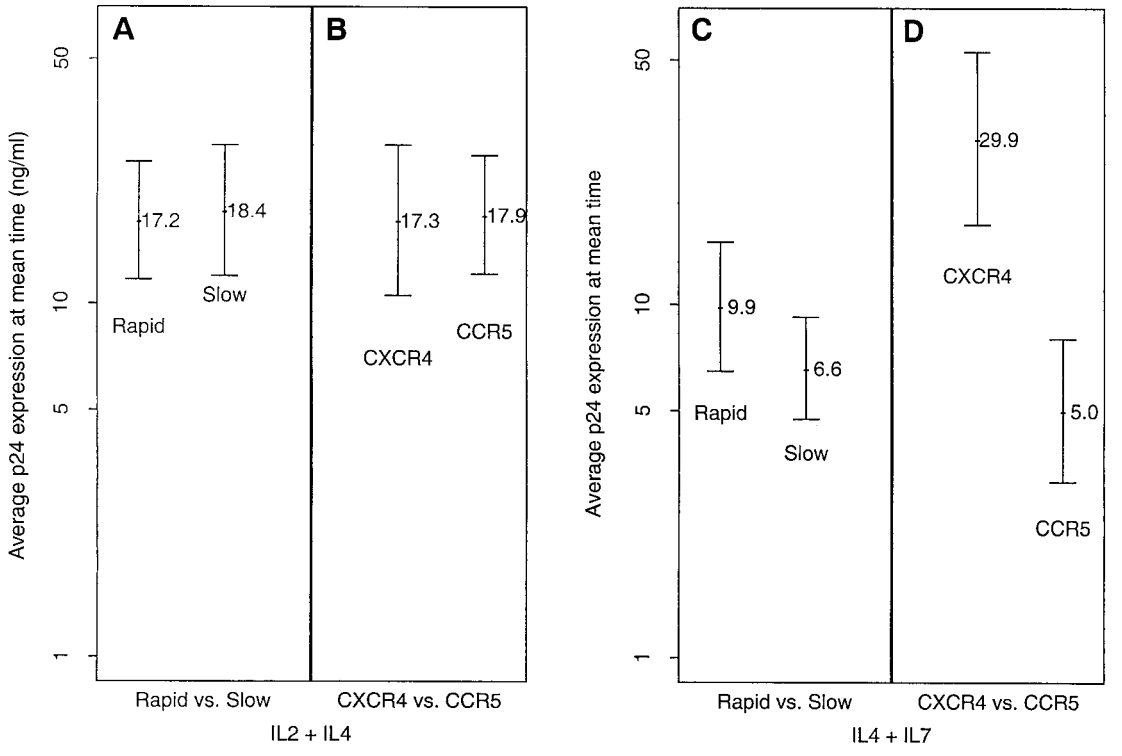

FIG. 5. Differential effects of IL-4 and IL-7 on the replication of HIV pediatric isolates in thymocytes. The estimated p24 production at the mid-point of the growth curves (ca. 2 weeks postinfection) was calculated for each isolate in at least three independent experiments, and then interexperiment comparisons were made between the different categories of pediatric isolates. In each experiment, freshly isolated thymocytes were infected in vitro with HIV pediatric primary isolates and cultured for 2 to 3 weeks in parallel with IL-2 (20 U/ml) and IL-4 (20 ng/ml) (A and B) or with IL-4 (20 ng/ml) and IL-7 (200 U/ml) (C and D). Viral production was measured for p24 antigen levels in the supernatant. The comparisons between isolates according to rate of disease progression (A and C) and coreceptor use (B and D) are shown. (The data in panels A and B include the results of the representative experiments shown in Fig. 1.) The difference in p24 levels is significant for both of the IL-4+IL-7 comparisons: the pediatric isolates from children with rapid disease progression showed higher production of p24 ($P = 0.05$) than the pediatric isolates from children with low rate of disease progression; the X4 PI isolates show a higher production than the R5 PI isolates ($P = 0.0001$).

cells expressing high levels of CXCR4 was lower in the presence of mitomycin C.

The effect of cytokines on CCR5 expression was further characterized. Only the combination of IL-2 and IL-4 reproducibly increased the number of $CD27^+$ thymocytes expressing CCR5 (see examples in Fig. 3B, 7B, and Table 4). In addition, we found that the $CD27^+$ $CCR5^+$ cells also expressed the differentiation marker CD44 and the activation marker CD25, which are present in <1% of freshly isolated postnatal thymocytes (Fig. 7B and data not shown). However, this $CCR5^+$ population was absent in thymocytes incubated with IL-2 and IL-4 in the presence of mitomycin C or HXU (Table 4). These results suggest that a subset of $CD3^{+high}$ thymocytes expressing CD27 and CCR5 can be activated and proliferate in response to cytokines, potentially increasing the number of targets for HIV productive infection. This may explain why CCR5, which is expressed at very low levels in the thymuses of HIV-negative infants and children, can support high levels of replication of R5 isolates in thymocytes.

DISCUSSION

In the present study we analyzed the role of chemokine receptors and cytokines in HIV infection of postnatal thymocytes, with 10 HIV-1 primary isolates from infants obtained at or close to birth. Our study shows that all primary isolates from infants were able to infect nonstimulated thymocytes freshly isolated from HIV-seronegative children. The primary isolates from neonates and infants studied here utilized CCR5, CXCR4, or both chemokine receptors to enter thymocytes. Neonatal X4 and R5 isolates were able to impact thymopoiesis. Surprisingly, the loss of CD4 expression in thymocytes infected with the different HIV isolates did not correlate with coreceptor use in the thymus and was not increased in children with a rapid rate of disease progression. This study also focused on the impact of cytokines involved in T-cell development on the replication of HIV primary isolates. IL-2, IL-4, and IL-7 did not prevent the loss of thymocytes observed during HIV infection and enhanced the replication of both X4 and R5 primary isolates in the thymus. However, our data suggest that HIV replication in thymocytes is differentially affected by distinct cytokine combinations of IL-2, IL-4, and IL-7. Although the replication kinetics of both X4 and R5 pediatric isolates in thymocytes was comparably enhanced in the combination IL-2+IL-4, IL-4 and IL-7 had a significantly higher impact on the replication kinetics of X4 pediatric isolates compared to the R5 isolates. Taken together, our data suggest multiple mech-

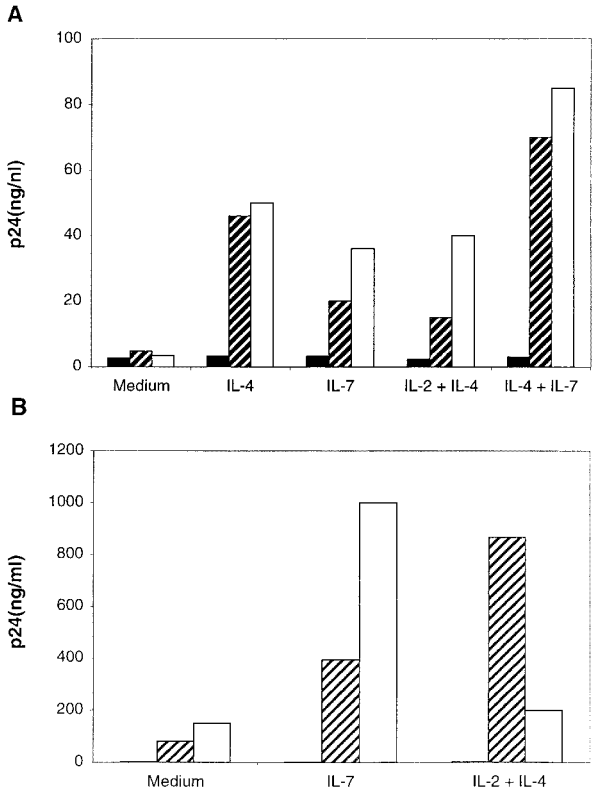

FIG. 6. Cytokines enhance HIV replication in the absence and presence of stromal elements. Nylon wool-purified thymocytes (A) and thymic organ cultures (B) were infected with the X4 isolate PI-2 and cultured in the presence or absence of the indicated cytokines for 2 weeks. (A) Thymocytes were infected in suspension and then cultured in serum-free medium in the presence of cytokines. Virus production was measured by enzyme-linked immunosorbent assay in the supernatants at day 5 (■), day 8 (▨), and day 11 (□) postinfection. (B) Thymus pieces were excised from a postnatal thymus, pooled for infection, and incubated with PI-2- or mock-infected supernatants. The thymus pieces were distributed at four pieces per raft in duplicate wells containing 3 ml of medium with or without the indicated cytokines. Virus production was measured in the supernatants of individual wells, and the average of two wells was determined at day 5 (■), day 10 (▨), and day 14 (□) postinfection.

anisms for IL-2, IL-4, and IL-7 enhancement of HIV replication, which are related to their ability to regulate coreceptor expression, differentiation, and proliferation of specific thymocyte subsets.

Coreceptor use by neonatal HIV-1 isolates in the thymus and disease progression. The wide distribution of CXCR4 in thymocytes suggested that vertical transmission of X4 variants could have a greater impact in thymopoiesis and thereby affect disease progression in children. Therefore, we determined coreceptor use by the neonatal HIV isolates by using compounds to block surface expression of chemokine receptors and viral replication directly in thymocytes. We found that the CXCR4-specific antagonist AMD3100 inhibited, totally or partially, the replication of three of six isolates from children infected in utero with a rapid rate of disease progression (Fig. 2). However, R5 isolates with rapid kinetics of replication in thymocytes were also present at birth in children infected in utero and in early samples from children infected intrapartum. This suggests that, in the thymus, perinatally transmitted HIV can replicate despite low levels of CCR5 expression, perhaps due to a high affinity for CCR5 (13, 43). Our observation is in agreement with the fact that heterozygosity for the CCR5 32-bp-deleted allele is found among perinatally infected children at the same frequency as in exposed noninfected children (17). Nonetheless, this mutation is present at a higher frequency among infected children with slow disease progression than in children with rapid disease progression (9). Recent studies found that among children heterozygous for the delta 32 mutation, those harboring X4 viruses (as defined by their ability to induce syncytia in the MT-2 assay) had a more rapid disease progression than those who did not (58). A rapid switch from CCR5 to CXCR4 use (or both coreceptors) in peripheral $CD4^+$ T cells has been suggested as a factor contributing to rapid disease progression in some children (61). By using the MT-2 assay and coreceptor-transfected HOS cells, no evidence of a phenotype switch from CCR5 to CXCR4 use was found in sequential viral isolates from the children with R5 isolates at birth and rapid disease progression studied here (Bryson et al., unpublished). It is possible that the R5 viruses obtained at birth from children with a more rapid rate of disease progression have a reduced sensitivity for C-C chemokines in vivo (61, 84) or that, in these children, the R5 isolates from later stages of disease became more virulent for thymocytes (66).

The HIV primary isolates studied here were not biologically cloned, although they were obtained from the HIV-positive cocultures with the highest serial dilution of the infant sample (14, 23). Analysis of the envelope (*env*) sequence diversity in the blood samples from mother-infant pairs from whom some of the isolates were obtained revealed relatively homogeneous quasispecies in children near birth (15, 23). For example, isolates such as PI-2, PI-5, and PI-8 were obtained from infants in whom transmission of a single maternal variant was detected, whereas isolates PI-1, PI-6, and PI-10 were obtained from infants in whom multiple variants from the mothers were found. However, minimal changes in the *env* gene may be sufficient to alter the affinity of HIV for CD4 and/or chemokine receptors and confer expanded or restricted use of coreceptors (69), especially in heterogeneous populations of primary cells such as thymocytes. Indeed, the presence of such variants within an isolate may explain why some isolates could use both chemokine receptors, although with different efficiencies (Fig. 2). In this regard, our results underscore the importance of using primary cells from different tissues to complement studies of coreceptor use in transfected cell lines. Subtle differences in expression, availability, and/or conformational changes in coreceptors among primary cells (13, 42) may result in tissue-specific use of coreceptors by HIV clinical isolates in vivo. Although the blocking studies to determine the tropism of pediatric isolates in the thymus show that CCR5 and/or CXCR4 are preferentially and efficiently used in vitro, they do not preclude the use of other chemokine receptors in vivo. The cytokines utilized in our system may affect the expression in vitro of other chemokine receptors present in the thymus, such as CCR4, CCR8, and CCR9, which could be used by HIV primary isolates in vivo. We have recently found that STLR33 surface expression levels in the thymus, which are as low as CCR5 levels in freshly isolated thymocytes, are not altered by the cytokines used in our system (data not shown). Finally, the

TABLE 4. Effect of proliferation on upregulation of chemokine receptors in thymocyte subsets[a]

Expt	Cytokine	Mitomycin C or HXU[b]	% CD27$^+$ cells	% CD27$^+$ CCR5$^+$ cells	% CD27$^+$ CXCR4$^+$ cells (CXCR4 MFI)[c]	% CD45RA$^+$ CXCR4$^+$ (CXCR4 MFI)[c]
1	None	−	5	<1	3 (57)	<1 (67)
	IL-2	−	37	1	26 (126)	11 (183)
		+	3	<1	3 (382)	2 (414)
	IL-4	−	42	<1	42 (973)	38 (1106)
		+	7	<1	6 (732)	3 (693)
	IL-7	−	62	<1	62 (547)	39 (761)
		+	17	<1	17 (278)	7 (461)
	IL-2+IL-4	−	68	8	68 (894)	40 (973)
		+	4	<1	4 (951)	2 (874)
	IL-4+IL-7	−	57	<1	56 (885)	44 (1090)
		+	10	<1	10 (681)	5 (827)
2	None	−	3	<1	3 (39)	ND
	IL-2	−	30	1	26 (165)	ND
		+	8	<1	7 (110)	ND
	IL-4	−	28	1	28 (667)	ND
		+	10	<1	10 (370)	ND
	IL-7	−	51	<1	51 (296)	ND
		+	14	<1	14 (108)	ND
	IL-2+IL-4	−	35	6	35 (587)	ND
		+	10	<1	10 (374)	ND
	IL-4+IL-7	−	39	<1	39 (571)	ND
		+	4	<1	4 (304)	ND

[a] Freshly isolated thymocytes were cultured for 2 weeks with the indicated cytokines in the presence or absence of the cell cycle inhibitors mitomycin C or HXU and analyzed by flow cytometry.

[b] In experiment 1, thymocytes were preincubated with (+) or without (−) mitomycin C (25 μg/ml) for 30 min, washed several times, and incubated with cytokines for 2 weeks in the absence of mitomycin C. In experiment 2, thymocytes were incubated with cytokines in the presence (+) or absence (−) of HXU (10 mM) throughout the experiment.

[c] Thymocytes were analyzed for thymocyte markers of maturation and chemokine receptors with the antibodies CD27-FITC, CD45RA-FITC, CCR5-PE (2D7), and CXCR4-PE (12G5). 7-AAD was used to discriminate apoptotic and dead cells. The percentage of live and early apoptotic cells is given for all subsets. The MFI of CXCR4 in the CD27$^+$ and CD45RA$^+$ thymocytes is shown in parentheses.

HIV pediatric isolates examined here were originally obtained from blood samples—and not from the thymus—by coculturing patient samples with PBMC or CD4$^+$-cell-enriched PBMC (14, 83). Therefore, in the initial process of virus isolation, viral species able to grow in thymocytes but not in PBMC could be counterselected. Since these are standard procedures for HIV isolation, this may also explain published data showing that most primary isolates use CCR5 and/or CXCR4, in addition to other coreceptors (reviewed and discussed in reference 3). Studies to explore those issues are currently under way in our laboratory.

Most pediatric HIV isolates are pathogenic in the thymus, regardless of coreceptor use. Association of early spontaneous abortions with fetal thymic abnormalities suggests that HIV variants able to wreak havoc in the thymus could be a determinant of the rapid rate of disease progression observed in children infected in utero (39, 54, 67). The timing of infection of the thymus in utero by X4 or R5 viruses may also contribute to thymic dysfunction since they infect different thymocyte subsets. However, we found that X4 primary isolates and most of the R5 HIV pediatric isolates induced loss of discrete subsets of thymocytes rather than a generalized disruption of thymopoiesis, despite the wide distribution of CXCR4 in the thymus (Fig. 4). A similar situation was described in the tonsil, where it was shown that R5 and X4 laboratory-adapted viruses are equally cytopathic for cells expressing the appropriate coreceptors (27). In the thymus, the cytopathic effect of HIV was not determined exclusively by use of specific coreceptors, since differences in the extent of CD4$^+$-cell loss were found among isolates using the same coreceptors (Fig. 3 and 4 and Table 3). This could be due to differences in the fusogenic capacity of the *env* gene of individual HIV pediatric isolates; to differences in the affinity of the isolates for CD4, CXCR4, and CCR5; or to the sensitivity of the different isolates to the effect of chemokines. Alternatively, differences in the cytopathic effect of individual pediatric isolates could be due to factors not related to viral entry and may reflect differences among their regulatory genes (such as *nef* and *tat*) or the long terminal repeats that result in an increased viral load. For example, *nef* is a determinant of HIV pathogenesis in the SCID-hu mouse (31) and the *nef* allele of the laboratory virus NL4-3 is itself cytopathic for thymocytes in vitro (79).

Cytokines enhance HIV replication in the thymus by inducing T-cell differentiation and expansion of mature thymocyte subsets expressing CXCR4 or CCR5. Cytokines may enhance HIV replication in the thymus by alleviating restrictions to viral replication at the postentry level in thymocyte subsets expressing appropriate coreceptors or by inducing coreceptor expression in subsets otherwise fully permissive for HIV replication (56). The data presented here suggest a dual effect of IL-2, IL-4, and IL-7 on HIV replication in the thymus that is related to changes in coreceptor expression and to the effect of cytokines on the activation, proliferation, and differentiation stages of the target cells. Indeed, the role of IL-4 on thymocyte maturation may explain why IL-4 downregulates CCR5 expression in lymphocytes (78) but not in thymocytes, where it synergizes with IL-2 to induce differentiation into and the expansion of a subset of activated CD27$^+$ CCR5$^+$ thymocytes (Fig.

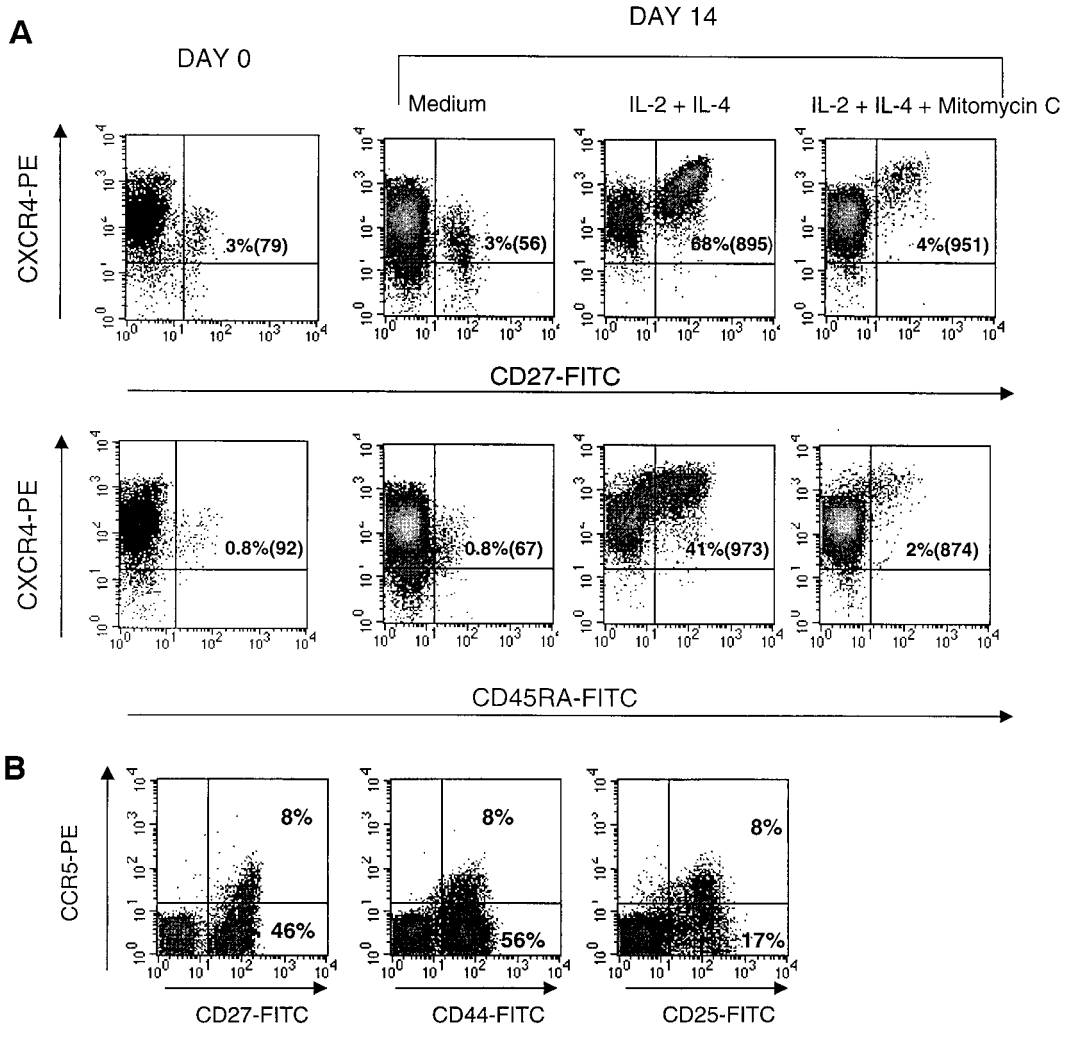

FIG. 7. Role of activation and proliferation on the upregulation of chemokine receptor expression by IL-2+IL-4. Freshly isolated thymocytes were preincubated with or without mitomycin C and cultured with IL-2+IL-4 for 2 weeks as described in Table 3. Before culture (day 0) and on day 14 after infection the cells were removed for assessment of chemokine receptor surface expression in different thymocyte subsets. Appropriate isotype control antibodies were used to set the cursors. Dead cells and apoptotic cells were identified by using 7-AAD. Density plots of thymocytes gated on live and early apoptotic cells are shown. (A) The percentages of $CD27^+$ and $CD45RA^+$ cells expressing CXCR4 and the MFI values of CXCR4 (in parentheses) in these populations are indicated. (B) The percentages of cells expressing CCR5 and the markers CD27, CD44, and CD25 are indicated. At 2 weeks after infection, <1% of the cells expressed CCR5 in the presence of mitomycin C (see Table 4).

4 and 7, Table 4). IL-4 and IL-7 upregulate CXCR4 but not CCR5 expression in this $CD27^+$ population, which expresses low levels of CXCR4 in vivo (Fig. 7). In addition, recent studies in our laboratory have shown that the presence of the $CD27^+$ population at the moment of infection is essential for virus replication in thymocytes in vitro (Gurney and Uittenbogaart, unpublished), further delineating the mature thymocyte subset in which we and others observe HIV expression (10, 28, 44, 56). More-recent studies with an X4 variant suggest that IL-7 favors the persistence of HIV in mature $CD4^+$ $CD8^-$ $CD3^+$ thymocytes, which we characterized here as being $CD27^+$ (28). Since CD27 interaction with its ligand, CD70, leads to NF-κB activation and tumor necrosis factor alpha secretion, this thymocyte subset may be well equipped to support postentry events of the virus replicative cycle (10, 86). Studies using retroviral vectors to bypass the influence of IL-7 on coreceptor expression suggest that this cytokine has a differential impact on HIV replication in neonatal and adult $CD4^+$ T cells (12). In our case, inhibition of cell cycle progression in the presence of cytokines leads to a reduction in the targets for HIV infection (Table 4) and to a lack of HIV expression (36, 74). HIV primary isolates may differ in their ability to productively infect thymocytes at distinct stages of activation and cell cycle progression, and isolates unable to replicate in nonoptimally activated cells could be responding differently to cytokines (18). Therefore, our studies and the data currently available in the literature suggest that enhancement of HIV replication by cytokines is related to their ability to increase targets for HIV infection by impacting both viral entry and later steps of HIV replication.

It is currently accepted that cytokines increasing thymic output and/or peripheral expansion of $CD4^+$ T cells could be used in conjunction with antiretroviral treatment to augment T-cell reconstitution (21, 51; reviewed in reference 59). In this regard,

it is noteworthy that antagonists of chemokine receptors, such as AMD3100 and chemokine receptor antibodies, were able to block the replication of HIV primary isolates in vitro despite upregulation of these HIV coreceptors induced by the cytokines. Our studies in the SCID-hu mouse show that in absence of antiretroviral treatment, the cytokines IL-4 and IL-7 modulated the loss of thymocytes observed during HIV infection but increased, rather than reduced, the viral load, whereas treatment with IL-2 does not increase viral load (75). Intermittent IL-2 therapy in HIV patients receiving highly active antiretroviral therapy (HAART) increased $CD4^+$-T-cell counts and reduced the pool of latently infected cells while inducing a transient increase in viremia, which coincides with the upregulation of CCR5 expression in vivo (11, 85). Therefore, studies defining how cytokines differentially control viral replication in the thymus may lead to therapeutic approaches to "tip the balance" in favor of increased T-cell reconstitution and reduced viral replication.

The main goal of this study was to investigate whether neonatal HIV-1 isolates from children with different timing of transmission and disease progression had a differential impact on the thymus. We were unable to establish a direct correlation between these clinical aspects and the replication kinetics or the pathogenic effects of neonatal HIV isolates in thymocytes. No correlation was found between coreceptor use in the thymus by HIV primary isolates and pathogenesis. Moreover, PI-8, an isolate highly adapted to the thymus in terms of replication kinetics, response to local cytokines, and possible extended coreceptor use was obtained from an infant infected intrapartum who remained asymptomatic until 7 years of age, with low levels of plasma viremia in the absence of antiretroviral treatment (reference 14 and data not shown). Therefore, individual host factors, which are keys to controlling disease progression in vertical transmission of HIV, remain to be established (5, 60, 81).

We propose that in the thymus cytokines may be a critical host factor in HIV pathogenesis given their role in thymopoiesis and their impact on HIV replication. Cytokine imbalances in the mother due to HIV infection (49, 68) may also alter T-cell development in utero and the susceptibility of thymocytes to infection, therefore influencing transmission and disease progression in the children. An inverse relationship between IL-7 levels and $CD4^+$-T-cell levels has been shown in HIV-infected individuals and in patients treated with chemotherapy, suggesting that IL-7 may play a role in T-cell homeostasis (21, 51). Since we showed that IL-7, as well as IL-4, has a differential effect on the replication of CXCR4-using and R5 isolates, increased levels of these cytokines in vivo may lead to selection of X4 variants in later stages of disease. This may be an additional factor operating in the observed correlation between maternal CD4 levels and increased HIV vertical transmission in the absence of therapy. In addition, HIV infection of the thymus per se could lead to the alteration of cytokine, chemokine expression, and the expression of their respective receptors, as was previously observed in other systems (26, 41, 53) and was recently seen in the thymus (35). Disruptions of peripheral cytokine networks have been reported in children exposed to HIV in utero and at birth (84). In conclusion, we found by using primary HIV isolates and examining the replication of HIV directly in thymocytes that host factors, such as cytokine levels, may be critical to the selection and transmission of HIV variants able to impact T-cell development. These results may be relevant for developing strategies of intervention in neonates and children that involve the use of cytokines for enhancing thymic output and immune reconstitution during HAART.

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